

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 39/12, 48/00, C07H 21/02, 21/04, C12N 5/10, 7/01, 7/04, 15/37, 15/86	A1	(11) International Publication Number: WO 00/09157 (43) International Publication Date: 24 February 2000 (24.02.00)
(21) International Application Number: PCT/US99/17931 (22) International Filing Date: 10 August 1999 (10.08.99) (30) Priority Data: 60/096,638 14 August 1998 (14.08.98) US (71) Applicant (for all designated States except US): MERCK & CO., INC. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LOWE, Robert, S. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). JANSEN, Kathrin, U. [DE/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). JOYCE, Joseph, G. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). MCCLEMENTS, William, L. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). COOK, James, C., III [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). LING, Jessica, Ching-Yee [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). NEEPER, Michael, P. [US/US]; 126 East Lincoln Avenue, Rahway, NJ 07065 (US).		(74) Common Representative: MERCK & CO., INC.; 126 East Lincoln Avenue, Rahway, NJ 07065 (US). (81) Designated States: AE, AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, ID, IL, IN, IS, JP, KG, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: PROTEIN DELIVERY SYSTEM USING HUMAN PAPILLOMAVIRUS VIRUS-LIKE PARTICLES (57) Abstract Human Papillomavirus virus like particles (VLPs) have been constructed so that they contain a modified L2 protein. The L2 protein has been minimized and is fused to a second protein or peptide. The fused protein is incorporated into the VLP and the VLP can deliver the protein to a cell. The modified VLPs can be used to increase the breadth of immune response in vaccine preparations or to deliver other proteins of interest.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

PROTEIN DELIVERY SYSTEM USING HUMAN PAPILLOMAVIRUS VIRUS-LIKE PARTICLES

FIELD OF THE INVENTION

5 This invention is related to a method of delivering protein to a cell by fusing it to a modified papillomavirus L2 protein, and expressing the fused protein in a virus-like particle. The invention is also directed to modified genes encoding the proteins, the modified virus-like particles and host cells containing the virus-like particles.

10

BACKGROUND OF THE INVENTION

 Human Papillomaviruses (HPVs) infect the genital tract and have been associated with various cancers and other diseases. Recombinantly produced L1 protein and L1 + L2 protein can self-assemble
15 to form virus-like particles (VLPs). These VLPs are immunogenic, and may be the basis of a vaccine.

 Currently, however there is no known prophylactic vaccine or ideal therapeutic treatment of disease caused by HPV. An immune
20 response to other HPV proteins co-delivered within the context of VLPs such as HPV early proteins or genes may enhance the protective effect of a vaccine. Recently, a paper by Greenstone et al described VLPs in which the L2 protein was modified to create a fusion protein between full length L2 and HPV E2 or E7. (Greenstone et al, 1998 *Proc. Natl. Acad. Sci. USA* 95:1800-1805.) The fusion proteins co-assembled with L1 into VLPs
25 which appeared indistinguishable morphologically from L1 VLPs and in their ability to elicit neutralizing antibodies. Furthermore, in tumor challenge models, mice vaccinated with chimeric L1 + L2:E7 fusion VLPs were found to protect mice from E7-expressing tumors.

 However, there are potential problems associated with the
30 use of a full-length L2 as a fusion partner. First, due to size restraints, an entire L2 as a fusion partner has a limited capacity to accept large fusion proteins. For instance, fusion of E1 with intact L2 of CRPV leads to morphologically aberrant VLPs. Furthermore, expression of a full-length L2 will most likely affect the activity of enzymatically active proteins and
35 therefore may limit its usefulness to deliver functional proteins into a cell. The minimal sequence requirements of L2 enabling coassembly with L1

into VLPs are unknown and the ability to delete significant portions of L2 while retaining the capacity to assemble with L1 is also unknown.

It would be desirable to provide a minimal L2 fusion protein which still retains the protein delivery function of a full-length L2, yet can
5 deliver larger and functionally-active proteins.

DESCRIPTION OF THE INVENTION

This invention is directed to a method of delivering a peptide or protein to a cell comprising the steps of: a) fusing a nucleic acid
10 sequence encoding the peptide or protein to be delivered to a modified papillomavirus (PV) L2 gene to create a fusion protein gene, wherein the L2 gene is less than full-length and comprises at least the coding sequences for the amino-terminal 69 amino acids and the carboxy-terminal 84 amino acids (aa) of L2; b) expressing the fusion protein gene
15 in a host cell to obtain a fusion protein; c) contacting the fusion protein with PV L1 protein under conditions wherein the fusion protein and the L1 protein spontaneously combine to form a virus-like particle (VLP); and d) delivering the VLP to a cell.

This invention also relates to nucleic acids encoding a fusion
20 protein comprising a less than full-length L2 protein fused to a gene encoding a peptide or protein of interest, and to these fusion proteins. In particular, this invention is related to L2 fusion proteins which comprise less than a full-length L2, and comprise at least the amino terminal 69 amino acids and the carboxy terminal 84 amino acids of L2. Additionally,
25 this invention relates to the virus-like particles (VLPs) which contain the fusion protein, and to cellular hosts containing these VLPs.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the assembly of the minimal L2 DNA construct
30 from native HPV16 L2. The coding sequence of HPV16 L2, shown in lower case italics, served as template for PCR amplifications. The initiator and terminator codons are in uppercase. Sense primers, I and A, are shown above the template; anti-sense primers, C and D, below. New sequence information,

introduced by PCR amplification are shown in uppercase. The complimentary sequences in primers A and C are underscored.

Figure 2 is the amino acid translation of the HPV 16 Minimal L2 Gene. Amino acid changes resulting from the introduction of the polylinker
5 containing unique restriction sites Not I, Sac I, and Xho I are indicated by italics. Amino acids 1-69 are contributed from the amino terminus of L2. A glutamic acid residue is added at position 70. Amino acids 71-154 are contributed from the carboxyl terminus of L2. At position 72, a glutamic acid residue replaces a serine found in the full-length wildtype L2.

10 Figure 3 is a Western blot of immunoprecipitated yeast lysates, showing co-immunoprecipitation of L2-E chimeric proteins with HPV16 L1 VLP-specific Mab H16:V5. The left panel shows anti-E1 stained immunoblot of minimal L2:E1 fusion (mL2:E1) clones E1-121 to E1-124, mL2:E2 sample E2-23 and no lysate samples are specificity
15 controls. The middle panel shows anti-E7 stained immunoblot of minimal L2:E7 fusion (mL2:E7) clones E7-125 to E7-127 and E7-31; mL2:E2 sample E2-23 and no lysate samples are specificity controls. The right panel shows anti-E2 stained immunoblot of mL2:E2 fusion clones E2-33a, E2-33b; sample E2-33b is also shown with no H16:V5 capture antibody as a control and YP3#1 no E protein control is shown as mL2 vector.
20

Figure 4 is a table listing beta galactosidase activities of transiently transfected cell lysates.

DETAILED DESCRIPTION OF THE INVENTION

25 The method of this invention can be used with virtually any papillomavirus strain. In preferred embodiments, the HPV is one of the strains which is associated with genital warts and/or genital cancers, and in particular may be selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV42,
30 HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.

Wild-type virus-like particles are predominantly made of L1 protein, although they contain a minor amount of L2 protein. In

accordance with this invention, the VLPs have been modified so that they still contain wild-type L1 protein, but now contain a deleted L2-fusion protein instead of wild-type L2. The L2 segment of the fusion protein is substantially less than the full length L2 protein; it contains peptide
5 domains from the amino terminus and from the carboxyl terminus which are necessary for association with L1 proteins and incorporation into VLPs, at the least, for HPV type 16, the coding sequences for the amino-terminal 69 amino acids and the carboxy-terminal 84 amino acids (aa) of L2. The L2 sequence may be larger than this, as long as it is less than
10 wild-type. Preferably the total amount of L2 amino acids present is less than about 60% of wild type, more preferably less than about 50% of wild-type, and even more preferably less than about 35% of wild type. This modified L2 will be referred to throughout the specification and claims as a "minimal L2" gene and protein.

15 Virtually any protein or peptide can be fused to the minimal L2 protein to make the fusion protein. HPV proteins which are normally not part of the VLP are preferred if the goal is to make a VLP which induces an increased immune response as compared to a wild-type VLP. For this application, these proteins include but are not restricted to HPV:
20 E1, E2, E3, E4, E5, E6, and/or E7, HIV TAT and/or beta lactamase. Particularly preferred proteins are E1, E2, and E7. These fusion proteins and the nucleic acids encoding them make up another aspect of this invention.

One of the advantages of this invention is that the fusion
25 partner may be a large protein. For example, the fusion partner may be over 50 kD, over 60 kD and even over 70 kD. This increased size allows for the introduction of most known proteins, and makes this delivery system particularly useful not only for HPV-related proteins as described above, but also for proteins which are not associated treatment of with
30 HPV disease.

Thus, this invention includes methods of using a modified VLP as a generalized transporter, directing the delivery of virtually any desired protein or peptide into cells. In one embodiment, reporter or regulatory proteins are delivered to cells as a measure of VLP uptake by
5 cells. As an example of fusion candidates which is by no means limiting, is the regulatory protein of HIV known as TAT. Fusions of TAT to the minimal L2 have been shown to retain protein function as well as form VLPs with L1. This is useful in establishing neutralization assays.

10 The fusion protein and L1 protein may be made simultaneously by transforming a selected host cell with genes encoding the proteins under conditions which allow expression of the two genes. General techniques, the genes for L1s and L2 , and methods for recombinant expression of L1 and L2 proteins are known in the art, and
15 may be used. Preferred host cells include yeast, insect cells, mammalian cells and *E. coli*.

Under one preferred embodiment of this invention, yeast are transformed with plasmid DNA containing the genes encoding L1 and the L2 fusion under control of known yeast promoters, such as the yeast Gal 1
20 and Gal 10 promoters. Expression of each gene product is induced by addition of galactose to the growth media, and the VLPs are isolated from the induced cell lysates.

Alternatively, the one set of host cells may be transformed to express only L1 protein, and a second set of host cells may be transformed to
25 express the L2 fusion protein of this invention. The respective proteins are harvested, optionally subjected to a purification step, and are brought into contact so that VLPs containing the fusion protein are formed.

The modified VLPs make up yet another aspect of this invention. These VLPs may be administered as a vaccine, or as part of a
30 therapeutic regimen. Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose PV infections. The effective amount may vary according to a

variety of factors such as the individual's condition, weight, gender and age. Other factors include the mode of administration. Generally, the compositions will be administered in dosages ranging from about 1 mcg to about 1 mg.

5 The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneously, topically, orally, mucosally, intravenously and intramuscularly.

 The vaccines of the invention comprise DNA, RNA or proteins encoded by the DNA that contain the amino-terminal and
10 carboxyl-terminal portions of L2 which permit the incorporation of proteins fused to it to be incorporated into VLPs. Such vaccines are also safe enough to be administered without danger of clinical infection, do not have toxic side effects, can be administered by an effective route, are stable, and are compatible with vaccine carriers.

15 The vaccines may be administered by a variety of routes, such as orally, parenterally, subcutaneously, mucosally, intravenously or intramuscularly. The dosage administered may vary with the condition, sex, weight, and age of the individual; the route of administration; and the type of PV of the vaccine. The vaccine may be used in dosage forms such
20 as capsules, suspensions, elixirs, or liquid solutions. The vaccine may be formulated with an immunologically acceptable carriers or adjuvants, or other excipients.

 The vaccines are administered in therapeutically effective amounts, that is, in amounts sufficient to generate a immunologically
25 protective response. The therapeutically effective amount may vary according to the type of PV. The vaccine may be administered in single or multiple doses.

 The purified proteins of the present invention may be used in the formulation of immunogenic compositions. Such compositions, when
30 introduced into a suitable host, are capable of inducing an immune response in the host.

The following non-limiting Examples are presented to better illustrate the invention.

EXAMPLE 1

5 Construction of the Modified L2 Gene

The modified HPV16 L2 gene was constructed from the coding sequences for the amino-terminal 69 amino acids and the carboxy-terminal 84 amino acids (aa) of HPV16 L2 which were fused in frame by a synthetic polylinker that introduced unique Not I, Sac I, and Xho I
10 restriction endonuclease recognition sites and resulted in the insertion of one glutamic acid residue and the mutation of a serine residue to glutamic acid. (Figure 1).

PCR primers (Midland Certified Reagents; Midland, TX)
15 were designed to amplify L2 sequences from the native L2 gene contained within the vector, pGal110 (Hofmann, K, et al 1995 *Virology* 209:506-518) into which the genes encoding HPV16 L1 +L2 were inserted. (See Figure 1).

Primers I (5'- CTT CCC CCC GGG CAC AAA ACA AAA
20 TGC-3'; SEQ.ID.NO. 1) and C (5'- CTC GAG CTC GCG GCC GCC TGT ACC CGA CCC-3'; SEQ.ID. NO. 2) amplified a 265 base pair (bp) sequence encoding the amino-terminal 69 aa and 23 bp of upstream untranslated sequence including a Sma I restriction enzyme site. Primer C modified and extended the L2 amino terminal-encoding region and
25 appended Not I, Sac I and Xho I restriction enzyme sites downstream of the L2-encoding sequences.

Primers A (5'- GCG GCC GCG AGC TCG AGG GTT ATA
TTC CTG CAA ATA CAA-3'; SEQ.ID.NO. 3), C and D (5'-CCC TCC AGA
TCT CTA GGC AGC CAA AGA GAC ATC TG-3' SEQ.ID.NO. 4) amplified
30 a 285 bp sequence encoding the carboxy-terminal 84 aa of L2 plus 6 bp which added a Bgl II restriction enzyme site. Primer A also appended a

17 bp sequence containing Not I, Sac I, and Xho I sites upstream of the L2-encoding sequence.

The minimal L2 expression construction was assembled through complementary sequences added by primers A and C. The isolated DNA products of the I/C and A/D amplification reactions above were both used in a PCR reaction which included the I and D primers. To facilitate the joining of the fragments through their 17 bp complementary sequence, three PCR cycles were performed with the annealing temperature at 37°C, followed by 15 cycles with the annealing temperature of 57°C. The resulting amplification product was blunt-end ligated into pcrScript (Stratagene, LaJolla, CA) and transformed into XL-1 Blue MRF' cells (Stratagene). Positive clones were identified by PCR using primers I and D, and confirmed by restriction digest analysis. The construction was then verified by automated sequence analysis (Perkin Elmer, Inc., Foster City, CA).

Plasmid DNA from an appropriate isolate was then digested with Sma I and Bgl II; a fragment of approximately 0.5 kilobase pairs (kb) was gel purified and ligated with the 14 kb Sma I and Bgl II vector fragment of pGAL110 into which the HPV16L1 gene was inserted adjacent to the GAL 1 promoter. Competent DH5 *E. coli* cells (Gibco BRL, Rockville, MD) were transformed with the ligation mixture and transformants selected on LB ampicillin plates (Remel, Lenexa, KS). Clones were initially screened by PCR in which primers D and I were used to amplify portions of L2. Sequencing of candidate clone YP3#1 verified the sequence to be that as shown in Figure 2.

YP3#1 was then employed as the backbone construct into which genes encoding HPV16 E1, E2, E7 or Tat open reading frames were inserted.

30

EXAMPLE 2

Insertion of HPV E Protein- and Tat-encoding Genes

The gene encoding HPV16 E2 was obtained by PCR amplification of a HPV16 positive clinical sample which was then inserted directly into the subcloning vector pCRII (Stratagene) and sequence verified as above. The E2 gene sequence was then modified in the following manner:

1) In-frame Xho I, Nae I, Not I -containing DNA sequences were added to the amino terminal portion of E2. Additionally, Not I, Nae I, and Xho I-containing sequences were added to the carboxyl-terminal portion of E2 to facilitate insertion within E2 at the Not I, Xho I sites.

2) The DNA sequences were altered by PCR mutagenesis to encode alanine residues encode at residues glutamic acid 39 and isoleucine 73. This was designed to inactivate E2 protein function.

The modified HPV16 E2 gene described above was digested with Not I, Xho I and ligated with similarly digested YP3#1 vector.

Transformants containing the properly-inserted E2 sequences were selected by PCR sequence verified and designated YP3-E2.

A similar approach was employed for inserting the genes encoding HPV16 E1, HPV16 E7 and HIV Tat (creating YP3-E1, YP3-E7 and YP3-Tat, respectively). The construct in which Tat was inserted at the carboxy terminus of minimal L2 was designated as YP3-cTat. For E1, glycine 482 was altered to aspartic acid; for E7, cysteine 24 and glutamic acid 26 were both changed to glycine to inactivate protein function. The resultant constructions were then used to transform yeast.

EXAMPLE 3

Identification and growth of yeast expressing chimeric VLPs

Plasmid DNA of YP3#1 and derivatives described above were used to transform *Saccharomyces cerevisiae* (*MAT α* , *leu2-04*, *prb1::HIS3*, *mn9::URA3*, *cir^o*) by the spheroplast method (Hinnen *et al.*, 1978, *Proc. Natl. Acad. Sci. USA* 75:1929-1933). Transformed spheroplasts were plated onto selective (leucine minus) medium (Remel, Lenexa, KS).

Clones were isolated through two rounds of single colony selection. Small liquid cultures of candidate clones were grown to high cell density in medium containing galactose. Crude extracts were prepared by vigorous agitation with glass beads followed by centrifugation. The clarified
5 extracts were analyzed for expression of L1, the L2 component, and VLPs by various methods including SDS PAGE, ELISA, immunoblotting, and EIA, using monoclonal antibodies or monospecific polyclonal antisera that recognize L1, or L2, or the amino or carboxy termini of L2, or L1 VLPs, or E1, or E2, or E7, or any other protein or peptide fused to the modified L2.
10 Clones which expressed the L2 component and formed VLPs were selected for further characterization. One-liter or 16-liter cultures of selected clones were grown in galactose containing medium for large-scale preparation of chimeric VLPs.

15

EXAMPLE 4

Purification of HPV Type 16 L1/L2_{mini}/E2 Chimeric VLPs

20 Cell pellets from a 16-liter fermentation of *S. cerevisiae* were stored frozen at -70°C. Frozen cells "Breaking Buffer" (200 mM MOPS, pH 7, 1 mM CaCl₂) was added to give approximately 20% (w/v) slurry. BENZONASE® (Nycomed Pharma) was added to 750 units/g wet cell weight. The cell slurry was broken at a pressure of approximately 19,000
25 psi by 5 passes in a M110-Y Microfluidizer (Microfluidics Corp., Newton, MA). The cell lysate was clarified by microfiltration through a 0.65 micron pore size hollow-fiber cartridge and then diafiltered with three volumes of 0.25 M sodium citrate, 0.2 M MOPS, pH 7.0. Permeate was loaded onto a POROS ® 50HS resin (Perseptive Biosystems, Cambridge,
30 MA) equilibrated in 200 mM MOPS, pH 7, 250 mM sodium citrate. The column was washed with 50 mM MOPS, 0.5 M NaCl, 5 mM sodium phosphate, pH 7 and eluted with a linear gradient from 0.5 to 1.5 M NaCl

in the same buffer. Column fractions were analyzed by immunoblotting and SDS-PAGE with colloidal Coomassie detection.

The 50HS pool was filtered through a 0.22 mm filter and applied to a ceramic hydroxyapatite (HA) Type II (Bio-Rad) column. The column was washed with 50 mM MOPS, pH 7, 1.25 M NaCl, 5 mM sodium phosphate and eluted with a linear gradient from 5 to 200 mM sodium phosphate, pH 7 in 1.25 M NaCl. Fractions were analyzed by Western blot and SDS-PAGE. Fractions showing comparable purity and enrichment of L1 protein were pooled. The pooled fractions were filtered aseptically through a 0.22 mm membrane and submitted for electron microscopy.

EXAMPLE 5

Detection of VLPs by Electron Microscopy

Transmission electron microscopy was performed by EMBS (Elkridge, MD). Appropriately diluted samples were placed on a 300 mesh carbon-coated copper grid and allowed to air dry. Grids were stained with phosphotungstic acid. All microscopy was performed using a JEOL 1200 EX transmission electron microscope. The micrographs generated had a final magnification of 138,000 x. Electron microscopy confirmed the presence of intact VLP particles with a mean diameter of 32 nm. These particles were indistinguishable morphologically from yeast-expressed L1 or L1+ L2 particles. Confirmation of the presence of L2 fusions within the VLPs was demonstrated by immunoblotting analysis of process fractions (See Example 6).

EXAMPLE 6

Co-immunoprecipitation of L2 fusion proteins with L1 specific anti-VLP antibodies.

To show that the L2 fusions were an integral component of the VLPs, VLPs were captured by an HPV16 L1 VLP-specific monoclonal antibody (mAb) H16:V5 (Wang, D. et al. 1997 *J. General Virology*, 78: 2209-2215), washed extensively and the retained products detected by immunoblotting. In brief, magnetic beads precoated with sheep anti-mouse IgG (Dynabeads M-280; Dynal, Oslo) were washed with TMOPS buffer (0.05M MOPS, pH 7.0, 0.4M NaCl, 0.1% Tween-80) containing 5% non-fat dry milk. The washed beads were incubated overnight at 4°C with H16:V5 mAb in the same solution. Unbound mAb was removed by washing with TMOPS. Beads were then incubated with control or VLP-containing crude yeast extracts (see Example 3) or partially-purified VLP preparations (see Example 4). Extracts containing 100-400 µg total protein were diluted with 0.2 MOPS, pH 7.0, 2 mM MgCl₂ and incubated with beads for 4 hours at 4°C. Beads were then washed extensively with TMOPS; bound proteins were recovered by heating the beads at 95°C in Laemmli sample buffer, resolved by SDS-PAGE and identified by immunoblotting.

Identification of the L2 fusion protein was performed with antibodies that recognize the L2 portion of the protein and by antibodies that recognize the protein of interest fused to L2, for example E1, E2, or E7. In addition to the inclusion of control yeast extracts, other controls included 1) beads which have not been incubated with H16:V5, and 2) beads which have not been incubated with yeast extract. Figure 3 shows the immunoblot results of VLPs captured by the H16:V5 Mab and immunoblotted as described above and then stained with anti-E1, anti-E7 or anti-E2 antisera. In each case for multiple isolates, the E-protein:L2 fusions were detected amount the captured, purified VLPs using the appropriate antibody, but not in the absence of lysate or using purified chimeric VLPs containing E-proteins different from the detection antisera.

Coprecipitation of the L2 fusion protein with L1 using a monoclonal antibody specific for L1 VLPs was taken as an indication that the L2 fusion protein was associated with the VLP.

EXAMPLE 7

Demonstration of Tat activity in Tat/minimal L2 fusions in mammalian cells.

5 To demonstrate that Tat /L2 fusions retained Tat activity, an expression plasmid was constructed in which DNA encoding the HIV Tat gene was inserted downstream of the minimal L2 gene so that it would be linked to the carboxyl terminus of L2 in the translated product. This DNA cassette was digested with Bgl II and inserted within the Bgl II site of the
10 mammalian expression vector, V1Jp (Montgomery, D. et al 1993 *DNA & Cell Biol.* 12:777) adjacent to the CMV promoter. The L2/Tat fusion was created by PCR of the minimal L2 (lacking a translational stop signal) using the oligomers 5'-TCC CCC GGG AGA TCT GCC ACC ATG CGA CAC AAA CGT TCT GCA AAA C- 3' (primer W; SEQ ID NO:5) and 5'-
15 GGC AGC CAA AGA GAC ATC TG-3' (primer X; SEQ ID NO:6). Oligomers 5'-CAG ATG TCT CTT TGG CTG CCA TGG AGC CAG TAG ATC CTA GAC-3'(primer Y; SEQ ID NO:7) and 5'-CTC GTA AGA TCT CTA TTC CTT CGG GCC TGT C-3'(primer Z; SEQ ID NO:8) were used to amplify the complete Tat open reading frame and to introduce sequence
20 complementarity with L2. The products from both PCR reactions were agarose gel isolated and then combined in a second PCR reaction in which only primers W and Z were added which generated the L2/Tat fusion through overlap of complementary sequences. These primers also appended Bgl II sites which were digested to facilitate insertion within
25 V1Jp to form V1Jp-L2cTat.

 Activity of the L2/Tat fusion was determined by transfection of V1Jp-L2cTat into P4R5 cells using a calcium phosphate transfection kit (available from Gibco BRL, Rockville, MD) performed as recommended by the manufacturer. P4R5 cells contain an integrated copy of the gene
30 encoding beta galactosidase under control of the HIV LTR. If the introduced Tat gene encodes functionally active Tat, expressed Tat protein will bind the LTR and result in expression of beta galactosidase. Lysates

were made of 48 hr post transfection cultures and beta galactosidase activity assessed using Galactostar® reagents (Tropix, Bedford, MS). The results are summarized in Figure 4 which shows that there were similar amounts of beta galactosidase produced in cells transfected with V1Jp-
5 L2cTat as with the unfused positive control pD5-Tat plasmid (235 vs. 175). The no-Tat DNA control had a value of 0.462. Thus, full Tat activity appears to be retained within the L2/Tat fusion.

WHAT IS CLAIMED IS:

1. A method of delivering a peptide or protein to a cell comprising:
 - a) fusing a first nucleic acid encoding the peptide or protein to a second nucleic acid encoding a modified human papillomavirus (HPV) L2 to create a fusion protein gene, wherein the L2 portion of the fusion protein gene is less than a full-length L2 gene, but encodes domains from the amino and the carboxyl termini;
 - b) expressing the fusion protein gene in a host cell to obtain fusion protein;
 - c) contacting the fusion protein with HPV L1 protein under conditions wherein the fusion protein and the L1 protein spontaneously combine to form a virus-like particle (VLP);
 - d) delivering the VLP to the cell.
2. A method according to Claim 1 wherein the HPV is selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.
3. A method according to Claim 2 wherein the L2 gene encodes less than about 50% of wild type L2 protein.
4. A method according to Claim 2 wherein the peptide or protein to be delivered is selected from the group consisting of: HPV E1, E2, E3, E4, E5, E6, E7, HIV Tat and beta-lactamase.
5. A nucleic acid encoding a fusion peptide or protein comprising: a first segment comprising a nucleic acid encoding a portion of an HPV L2 protein which is less than a full-length sequence, and a second segment comprising a nucleic acid encoding a peptide or protein.

6. A nucleic acid according to Claim 5 wherein the first segment is from an HPV selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35,
5 HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.

7. A nucleic acid according to Claim 6 wherein the second segment encodes a protein selected from the group consisting of: E1, E2, E3, E4, E5, E6, E7 HIV Tat and beta-lactamase.

10

8. A HPV virus-like particle (VLP) comprising HPV L1 protein and a fusion protein wherein the fusion protein comprises:

15 a first protein which is an HPV L2 protein which is less than about 50% of wild type; and

a second protein or peptide.

9. A VLP according to Claim 8 wherein the HPV is selected from the group consisting of: HPV6a, HPV6b, HPV11, HPV16,
20 HPV18, HPV31, HPV33, HPV35, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, and HPV56.

10. A host cell comprising the VLP of Claim 8.

25 11. A vaccine comprising a virus-like particle according to Claim 10.

12. A nucleic acid encoding a VLP according to Claim 9.

30

1/4

Sma I

primer I >cttcccccgggcacaaaacaaa
cttcccccgggcacaaaacaa

atgc>

ATGcgacacaaaacgttctgcaaaacgcacaaaacgtgcatcggctacccaactttataaa
acatgcaaacaggcaggtacatgtccacctgacattatacctaagggtgaaggcaaaact
attgctgatcaaatattacaatatggaagtatgggtgtatTTTTTgggtgggttaggaatt
ggaacagggtcgggtacaggcggacgcactgggtatattccattgggaacaaggcctccc
<cccagcccattgtcccccGgcqCTCGAGCTC< primer C

Not I Sac I

Xho I

acagctacagatacacttgctcctgtaagacccccctttaacagtagatcctgtgggccct
tctgatccttctatagtttcttttagtggaagaaactagttttattgatgctggtgcacca
acatctgtaccttccatccccccagatgtatcaggatttagtattactacttcaactgat
accacacctgctatattagatattaataactgttactactgttactacacataataat
cccacttttactgacccatctgtattgcagcctccaacacctgcagaaactggagggcat
tttacactttcatcatccactattagtagacataattatgaagaaattcctatggataca
tttattgttagcacaaaccctaacacagtaactagtagcacaccataaccaggtctcgc
ccagtggcacgcctaggattatagtcacacaacacaacaagttaaagttgtagaccct
gcttttgaaccactcccactaaacttattacatatgataatcctgcatatgaaggata
gatgtggataatacattatattttcctagtaataatgataatagtagtataatagctccagat
cctgactttttggatatagttgctttacataggccagcattaacctctaggcgtactggc
attaggtacagtagaattggtaataaacaacactacgtactcgtagtggaaaatctata
ggtgctaaggtagattattattatgatttgagtactattgatcctgcagaagaaatagaa
ttacaaactataacaccttctacatatatactaccacttcacatgcagcctcacctacttct
attaataatggcttatatgatatttatgcagatgactttattacagatacttctacaacc

Not I Sac I

Xho I

primer A >GCGGCCGCGAGCTCGAGgggttatattcctgcaaatacaac>
ccggtaccatctgtaccctctacatctttatcaggttatattcctgcaaatacaacaatt
ccttttgggtggtgcatacaaatattccttttagtatcaggctcctgatataccattaatata
actgaccaagctccttcattaattcctatagttccagggctccacaatatacaattatt
gctgatgcaggtgactttttttacatcctagttattacatgttacgaaaacgacgtaaa
cgtttaccatattttttttcagatgtcttttggtgccTAG

primer D <gtctacagagaaaccgacggatcTCTAGACCTCCC<

Bgl II

FIG. 1

2/4

```

ATG CGA CAC AAA CGT TCT GCA AAA CGC ACA AAA CGT GCA TCG GCT ACC CAA CTT
M  R  H  K  R  S  A  K  R  T  K  R  A  S  A  T  Q  L
1                                10
TAT AAA ACA TGC AAA CAG GCA GGT ACA TGT CCA CCT GAC ATT ATA CCT AAG GTT
Y  K  T  C  K  Q  A  G  T  C  P  P  D  I  I  P  K  V
20                                30
GAA GGC AAA ACT ATT GCT GAT CAA ATA TTA CAA TAT GGA AGT ATG GGT GTA TTT
E  G  K  T  I  A  D  Q  I  L  Q  Y  G  S  M  G  V  F
40                                50
Not I.  Sac I  Xho I
TTT GGT GGG TTA GGA ATT GGA ACA GGG TCG GGT ACA GGC GGC CGC GAG CTC GAG
F  G  G  L  G  I  G  T  G  S  G  T  G  G  R  E  L  E
60                                69 70 72
GGT TAT ATT CCT GCA AAT ACA ACA ATT CCT TTT GGT GGT GCA TAC AAT ATT CCT
G  Y  I  P  A  N  T  T  I  P  F  G  G  A  Y  N  I  P
80                                90
TTA GTA TCA GGT CCT GAT ATA CCC ATT AAT ATA ACT GAC CAA GCT CCT TCA TTA
L  V  S  G  P  D  I  P  I  N  I  T  D  Q  A  P  S  L
100
ATT CCT ATA GTT CCA GGG TCT CCA CAA TAT ACA ATT ATT GCT GAT GCA GGT GAC
I  P  I  V  P  G  S  P  Q  Y  T  I  I  A  D  A  G  D
110                                120
TTT TAT TTA CAT CCT AGT TAT TAC ATG TTA CGA AAA CGA CGT AAA CGT TTA CCA
F  Y  L  H  P  S  Y  Y  M  L  R  K  R  R  K  R  L  P
130                                140
TAT TTT TTT TCA GAT GTC TCT TTG GCT GCC TAG
Y  F  F  S  D  V  S  L  A  A  .
150                                154

```

FIG. 2

3/4

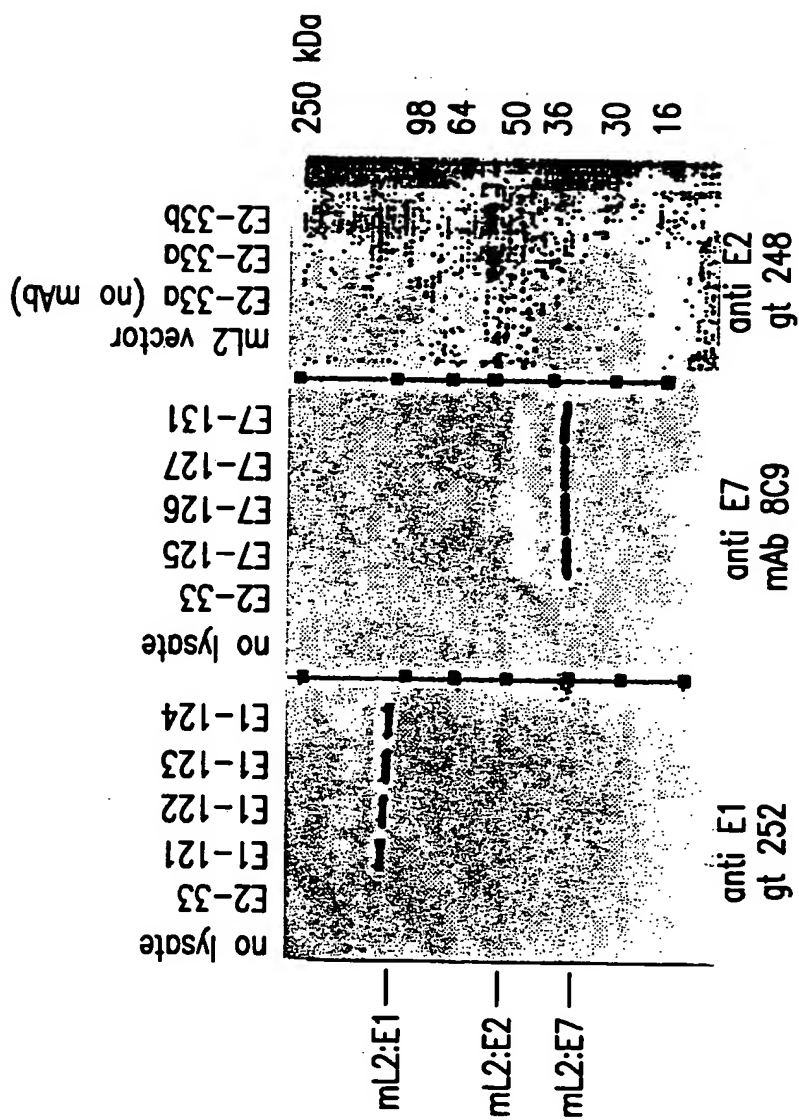


FIG.3

4/4

BETA-GALACTOSIDASE ACTIVITIES OF P4R5 CELL LYSATES TRANSFECTED WITH THE LISTED PLASMID CONSTRUCTS AND EVALUATED USING THE GALACTOSTAR [®] ASSAY	
SAMPLE	BETA-GALACTOSIDASE RELATIVE LIGHT UNITS
V1Jp-L2cTot	229.44
pD5 Tot (POSITIVE CONTROL)	196.48
MOCK TRANSFECTION (NEGATIVE CONTROL)	0.40

FIG.4

SEQUENCE LISTING

<110> Merck & Co., Inc.

<120> PROTEIN DELIVERY SYSTEM USING HUMAN
PAPILLOMAVIRUS VIRUS-LIKE PARTICLES

<130> 20276 PCT

<150> 60/096,638

<151> 1998-08-14

<160> 8

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 1

cttccccccg ggcacaaaac aaaatgc

27

<210> 2

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 2

ctcgagctcg cggccgcctg taccgaccc

30

<210> 3

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 3

gcggccgcga gctcgagggt tatattcctg caaatacaa

39

<210> 4

<211> 35

<212> DNA

<213> Artificial Sequence

<220>
<223> PCR primer

<400> 4
ccctccagat ctctaggcag ccaaagagac atctg 35

<210> 5
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 5
tcccccgga gatctgccac catgcgacac aaacgttctg caaaac 46

<210> 6
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 6
ggcagccaaa gagacatctg 20

<210> 7
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 7
cagatgtctc ttggctgcc atggagccag tagatcctag ac 42

<210> 8
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer

<400> 8
ctcgtaagat ctctattcct tcgggcctgt c 31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17931

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : 424/204.1, 93.2, 93.6; 514/2, 44; 435/455, 456, 320.1, 325, 366

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/204.1, 93.2, 93.6; 514/2, 44; 435/455, 456, 320.1, 325, 366

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, Dialog, Medline, Biosis, Biotech

Search terms: papillomavirus, L2, fusion, vaccine, L1, virus-like particle

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,618,536 A (LOWY et al.) 08 April 1997, columns 7-8 and claims 1-10.	5-7
A	US 5,437,951 A (LOWY et al.) 01 August 1995, columns 3-4.	1-12
A,P	US 5,855,891 A (LOWY et al.) 05 January 1999, columns 5-6.	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 20 DECEMBER 1999	Date of mailing of the international search report 03 FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>David Guzo</i> DAVID GUZO Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17931

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/12, 48/00; C07H 21/02, 21/04; C12N 5/10, 7/01, 7/04, 15/37, 15/86